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Expression Cloning of a Functional Glycoprotein Ligand for P-Selectin

Dianne Sako,* Xiao-Jia Chang,* Karen M. Barone,* Gloria Vachino,* Holly M. White,* Gray Shaw,* Geertruida M. Veldman,* Kevin M. Bean,* Tim J. Ahern,* Bruce Furie,† Dale A. Cumming,* and Glenn R. Larsen*

*Genetics Institute
Small Molecule Drug Discovery Group
87 CambridgePark Drive
Cambridge, Massachusetts 02140
†Center for Hemostasis and Thrombosis Research
Division of Hematology and Oncology
New England Medical Center
and the Department of Medicine
Tufts University School of Medicine
Boston, Massachusetts 02111

Summary

The initial adhesive interactions between circulating leukocytes and endothelia are mediated, in part, by P-selectin. We now report the expression cloning of a functional ligand for P-selectin from an HL-60 cDNA library. The predicted amino acid sequence reveals a novel mucin-like transmembrane protein. Significant binding of transfected COS cells to P-selectin requires coexpression of both the protein ligand and a fucosyltransferase. This binding is calcium dependent and can be inhibited by a neutralizing monoclonal antibody to P-selectin. Cotransfected COS cells express the ligand as a homodimer of 220 kd. A soluble ligand construct, when coexpressed with fucosyltransferase in COS cells, also mediates P-selectin binding and is immunocrossreactive with the major HL-60 glycoprotein that specifically binds P-selectin.

Introduction

Specific cell adhesion events, such as neutrophil binding to activated endothelia and platelets, are mediated in part by P-selectin (PADGEM, GMP-140, CD62). P-selectin is an integral membrane glycoprotein that is retained within storage granules of platelets and endothelial cells and rapidly translocated to the cell surface after appropriate stimuli (Stenberg et al., 1985; Berman et al., 1986). At the cell surface, P-selectin acts as a receptor for monocytes and neutrophils (Larsen et al., 1989; Hamburger and McEver, 1990) and can, for example, induce the tethering of circulating leukocytes to the blood vessel wall (Lawrence and Springer, 1991). P-selectin is one member of a family of homologous cell adhesion molecules that includes E-selectin (Bevilacqua et al., 1987) and L-selectin (Lasky et al., 1989).

The identity of the cognate ligands for the selectins is not completely understood. As is typical for many lectins, the selectins are promiscuous with regard to ligand specificity; a variety of carbohydrate ligands have been identified for each selectin (for a review, see Varki, 1992). The diversity of oligosaccharide structures bound by each selectin has led to questions concerning the identity of physiologically relevant ligands. For example, numerous mono- and oligosaccharides bind to L-selectin, yet biochemical studies have suggested that peripheral lymph nodes possess only two specific glycoproteins that present carbohydrate ligands (Lasky et al., 1992). Similar observations of a specific glycoprotein ligand for murine E-selectin have recently been reported (Levinovitz et al., 1993).

Several lines of evidence suggest that the physiological ligand for P-selectin is also a glycoprotein. The binding of myeloid cells to P-selectin is protease sensitive (Moore et al., 1991; Larsen et al., 1992), and pretreatment of HL-60 cells with tunicamycin, an inhibitor of protein N-glycosylation, significantly inhibits the adhesion of treated cells to CHO cells expressing P-selectin (Larsen et al., 1992). In addition, blotting of HL-60 membrane extracts with P-selectin has yielded a specific glycoprotein ligand (Moore et al., 1992). Thus, while P-selectin binds carbohydrate ligands such as 3-sialyl-Lewis x (SLe^x) (Larsen et al., 1990; Polley et al., 1991), it is likely that these or related carbohydrate moieties are presented on a polypeptide backbone.

We have explored the possibility that the physiological ligand for P-selectin may be a glycoprotein by expression cloning. Using this approach, we have identified a unique functional glycoprotein ligand for P-selectin.

Results

Expression Cloning of a P-Selectin Glycoprotein Ligand

Our expression cloning strategy employed COS cells cotransfected with an HL-60 cDNA library and a second plasmid, pEA.3/4FT, containing a specific fucosyltransferase cDNA. The rationale for this strategy was as follows. COS cells do not bind P-selectin nor do they possess the appropriate glycosylation apparatus to synthesize Lewis^x (Le^x) or SLe^x, presumed carbohydrate components of a P-selectin ligand (Larsen et al., 1990; Polley et al., 1991). However, COS will express SLe^x and Le^x when transfected with the cDNA encoding the α(1,3/1,4)fucosyltransferase (3/4FT) gene (Lowe et al., 1990). Moreover, we have shown previously that COS cells expressing the 3/4FT gene bind to CHO cells expressing E-selectin, but not P-selectin (Larsen et al., 1992). Thus, COS cells expressing 3/4FT could presumably allow the appropriate modification of proteins derived from a cotransfected HL-60 cDNA library.

Cotransfected COS cells were subjected to sequential rounds of panning as described in the Experimental Procedures. This procedure was pursued until a single plasmid (pPL85) was isolated, which, when cotransfected into COS cells with pEA.3/4FT, induced the binding of CHO–P-selectin, whereas CHO–P-selectin cells did not adhere to COS cells cotransfected with pEA.3/4FT and vector alone (data

not shown). Therefore, P-selectin-dependent binding was due to the expression of a glycoprotein, henceforth referred to as P-selectin glycoprotein ligand-1 (PSGL-1).

Sequence Analysis of PSGL-1 Indicates No Significant Homology to Other Proteins

The nucleotide sequence of the PSGL-1 cDNA in pPL85 is shown in Figure 1A. The cDNA encodes 1649 nt containing a putative translational start site at nucleotide 60, followed by a single open reading frame of 402 amino acids. The initiating methionine is followed by a putative 18 amino acid signal sequence containing a hydrophobic core (Figure 1B). Three potential sites for N-glycosylation are evident in the extracellular domain, which also exhibits regions with a high frequency of proline, serine, and threonine, including 15 consecutive repeats of the ten amino acid consensus sequence A-T/M-E-A-Q-T-T-X-P/L-A/T (Figure 1C), where X can be either P, A, Q, E, or R. A tetrapeptide consensus sequence (R-N-R-R) for cleavage by paired basic amino acid converting enzymes (PACE; Rehemtulla and Kaufman, 1992) is evident starting at position 38. Three potential sites of tyrosine sulfation are observed at positions 46, 48, and 51 (Huttner and Baeuerle, 1988). The remaining C-terminal sequence contains a putative transmembrane domain (residues 309–333), as determined by the method of Hopp and Woods (1981), and a cytoplasmic tail (residues 334–402).

Comparisons of the DNA and amino acid sequences of PSGL-1 to data base sequences failed, with a single exception, to identify any significant homology to other proteins, including the recently cloned ligand for L-selectin (Lasky et al., 1992). The only homology identified was a 27 amino acid portion spanning the putative transmembrane region of PSGL-1, beginning at residue 310, that is 48% identical to residues 125–151 of human *EVI2A*, the human homolog of a mouse gene implicated in leukemogenesis (Cawthon et al., 1990). Thus, PSGL-1 appears to be a unique, mucin-like membrane glycoprotein.

P-Selectin Binds Selectively to Cotransfected COS Cells Expressing PSGL-1

Figure 2 illustrates that COS cells transfected with either pPL85 (PSGL-1), pEA.3/4FT (fucosyltransferase), or vector alone fail to bind CHO–P-selectin cells. Significant binding is observed only when pPL85 and pEA.3/4FT are cotransfected, thus indicating that functional P-selectin binding activity requires appropriate glycosylation of PSGL-1. The adhesion of CHO–P-selectin cells to cotransfected COS cells was abolished in the presence of EGTA and EDTA; P-selectin-mediated cell adhesion is calcium

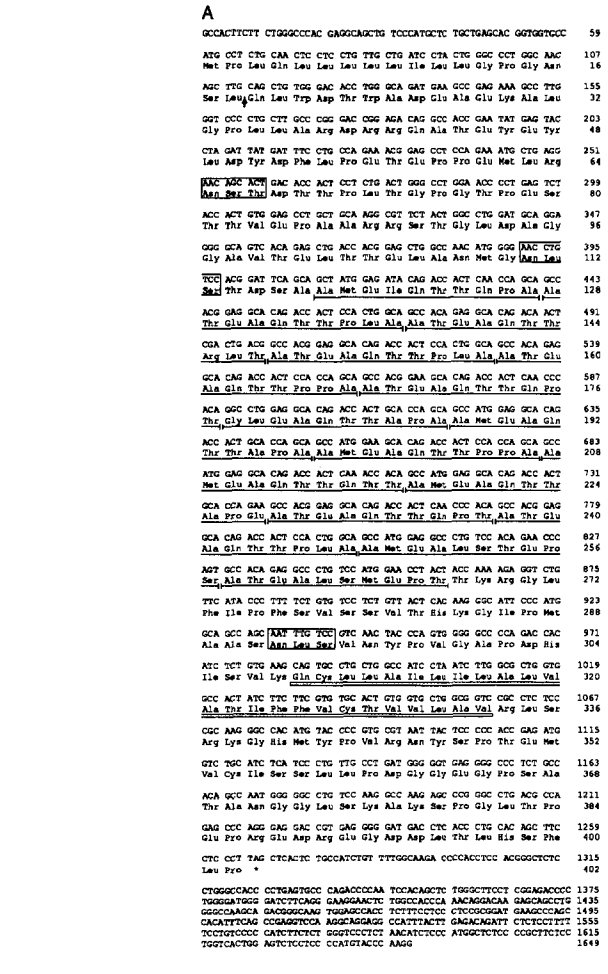

Figure 1. DNA, Amino Acid Sequence, and Protein Hydropathy Determination of the Human PSGL-1

(A) The nucleotide and inferred amino acid sequence of the P-selectin glycoprotein ligand. The arrow indicates the putative signal sequence cleavage site, determined by the method of von Heijne (1987); potential N-linked glycosylation sites are boxed; the 15 decameric repeats beginning at Alanine 118 are underlined; and the putative transmembrane domain is double underlined. (B) The hydropathic plot of PSGL-1 according to the method of Kyte and Doolittle (1982). Various regions are bracketed and identified at the right of the figure. Circled N's represent potential N-glycosylation sites. (C) Alignment of the decameric peptide repeats of PSGL-1.

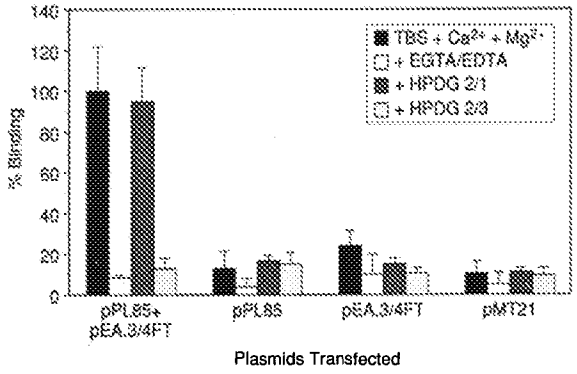

Figure 2. Specificity of CHO–P-Selectin Binding to COS Cells Expressing PSGL-1

COS cells were transfected with the plasmids as indicated. Prior to the adhesion assay, the CHO–P-selectin cells were incubated in binding buffer (closed bars), or binding buffer plus 3 mM EGTA and 3 mM EDTA (open bars), or binding buffer with either the nonneutralizing anti-P-selectin MAb HPDG 2/1 (dark stippled bars) or the neutralizing MAb HPDG 2/3 (light stippled bars).

dependent (Larsen et al., 1989; Hamburger and McEver, 1990). Heterotypic cell binding was also inhibited by the neutralizing anti-P-selectin monoclonal antibody (MAb HPDG 2/3) but not by the nonneutralizing anti-P-selectin MAb of the same isotype (MAb HPDG 2/1).

Membrane extracts prepared from cotransfected COS cells were characterized by protein blotting analysis using a chimeric form of P-selectin fused to a human Fc ($LEC_{\gamma 1}$) to assess the size of the recombinant protein. Two bands were observed by nonreducing SDS–polyacrylamide gel electrophoresis (PAGE). The major band migrated with an estimated molecular mass of 220 kd, whereas the minor band migrated with an approximate molecular mass of 110 kd (Figure 3A, lane 1). Under reducing conditions only a single band was observed with a molecular mass of approximately 110 kd (Figure 3B, lane 1), suggesting that under nonreducing conditions PSGL-1 exists as a homodimer. No specific bands were observed using human immunoglobulin G1 (IgG1) as an isotype control (Figure 3C, lane 1), and the binding of $LEC_{\gamma 1}$ to the 110 kd band was abrogated in the presence of EDTA and EGTA (Figure 3D, lane 1).

PSGL-1 Is Encoded by a Single-Copy Gene and Is Expressed in a Variety of Human Cells That Bind P-Selectin

Southern blot analysis of human genomic DNA using a 330 bp probe from the PSGL-1 cDNA clone (nucleotides 60–389) revealed a single hybridizing fragment in all DNA digests (Figure 4A), which was evident under low stringency conditions. This suggests that the PSGL-1 gene is present as a single copy in the human genome and is not a member of a multigene family.

Total or poly(A)$^+$ RNA isolated from various human cell types was examined by Northern blot analysis for the presence of PSGL-1 transcripts (Figure 4B). A prominent PSGL-1 transcript of approximately 2.5 kb (indicated by the closed arrow in Figure 4B) was observed in mRNA blots of HL-60 cells as well as the monocytic cell lines THP-1 and U937. PSGL-1 mRNA of the same size was detected in total RNA derived from freshly isolated monocytes and polymorphonuclear cells (PMNs). In addition, weak hybridization with an approximately 4 kb mRNA was observed in PMNs (Figure 4B, open arrow). We did observe the 4 kb mRNA in HL-60 and U937 cells upon longer exposure (data not shown) but failed to observe this signal in THP-1 and monocyte mRNA preparations. All of these cell types exhibit binding to CHO–P-selectin. Thus, the presence of PSGL-1 mRNA correlates with P-selectin binding in these cells. In contrast, no transcripts were detected in mRNA from the human hepatoblastoma cell line HepG2.

A Soluble Form of PSGL-1 Binds P- and E-Selectin

To demonstrate that the P-selectin binding properties of cotransfected COS cells are solely attributable to PSGL-1, a plasmid encoding a soluble form (sPSGL-1.T7) was constructed and expressed in COS cells. The sPSGL-1.T7 construct encodes the amino-terminal 295 residues of PSGL-1 fused to 46 additional residues that contain a ten amino acid epitope from the major capsid protein of the

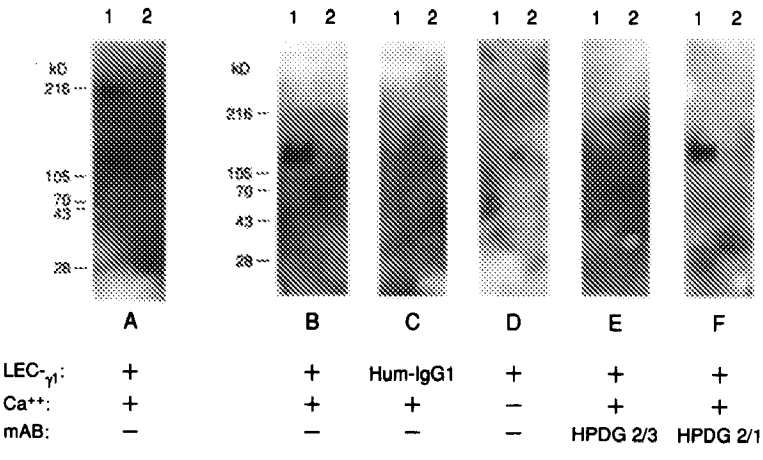

Figure 3. Protein Blot Analysis of a P-selectin IgG Chimera to COS Cell Extracts Expressing PSGL-1

Membrane proteins from COS cells cotransfected with pPL85 and pEA.3/4FT (lane 1) or pMT21 (control vector) and pEA.3/4FT (lane 2) were analyzed on a 6% nonreduced (A) or 8% reduced (B–F) SDS–PAGE. Protein blots were treated with ^{125}I-protein A–$LEC_{\gamma 1}$ (or human IgG1) under the conditions indicated at the bottom of each blot.

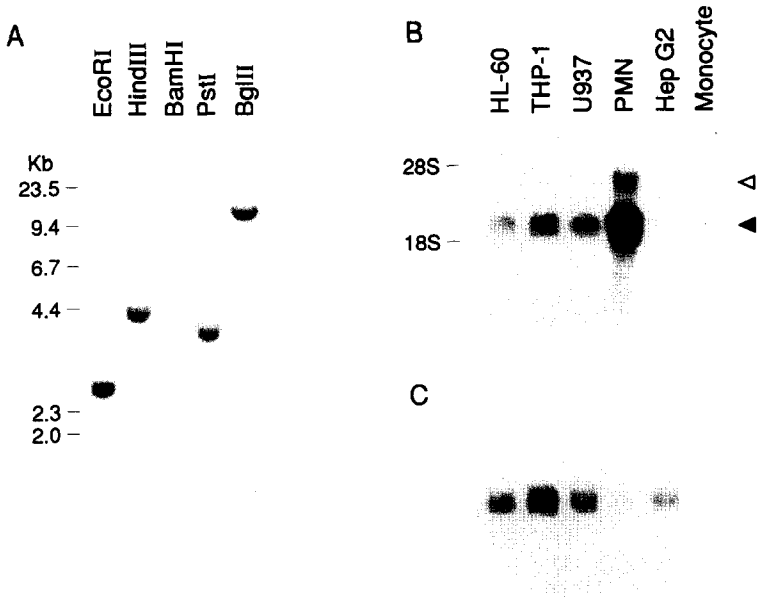

Figure 4. Southern and RNA Blot Analysis

(A) A ^{32}P-labeled probe comprising nucleotides 60–389 of PSGL-1 was hybridized under low stringency conditions with a human genomic DNA blot. Northern blots containing RNA prepared from HL-60, THP-1, U937, HepG2 cells (3 μg of poly(A)$^+$ RNA), human monocytes, and PMNs (10 μg total RNA) were hybridized under high stringency conditions with the same PSGL-1 probe (B) or a probe for actin (C).

bacteriophage T7. This epitope is recognized by the MAb T7 (Lutz-Freyermuth et al., 1990).

Conditioned medium from cotransfected COS cells was coated onto plates, and the adherence of radiolabeled CHO–P-selectin cells was measured. As shown in Figure 5A, CHO–P-selectin cells bound to plates coated with medium from COS cells cotransfected with pED.sPSGL-1.T7 and pEA.3/4FT, whereas the parental CHO-DUKX cells did not bind. As expected, conditioned medium from COS cells transfected with either pED.sPSGL-1.T7 or pEA.3/4FT alone failed to bind any CHO–P-selectin (Figure 5A).

The specific binding of sPSGL-1.T7 to P-selectin was again confirmed by two experiments. CHO–P-selectin cells failed to bind medium from cotransfected COS cells upon the addition of EDTA and EGTA, and a neutralizing MAb to P-selectin blocked this binding while a nonneutralizing MAb did not (data not shown). Thus, sPSGL-1.T7 possesses all of the P-selectin binding characteristics attributed to PSGL-1.

Conditioned medium generated from COS cells cotransfected with pED.sPSGL-1.T7 and pEA.3/4FT was also observed to direct the binding of CHO–E-selectin cells, but not the binding of parental CHO-DUKX cells (Figure 5A). Transfection of COS cells with either pED.sPSGL.T7 or pEA.3/4FT alone yielded conditioned medium incapable of supporting CHO–E-selectin binding (Figure 5A). Additional experiments demonstrated that binding is mediated by the lectin domain of E-selectin. The addition of EGTA and EDTA abolished CHO–E-selectin binding to conditioned medium from cotransfected COS cells (Figure 5B), as did an E-selectin-specific neutralizing MAb, HEL3/2 (Larsen et al., 1992) (Figure 5C).

Other Neutrophil Sialoglycoproteins Cotransfected into COS Cells Do Not Bind P-Selectin

To address the possibility that the polypeptide of PSGL-1 is merely an irrelevant scaffolding for the presentation of SLe^x to E- and P-selectin, further cotransfection experiments were performed to generate cell surface CD43 (leukosialin; Maemura and Fukuda, 1992) and L-selectin (Picker et al., 1991), two candidate selectin ligands. Figure 6 shows that only cotransfection with plasmids encoding 3/4FT and PSGL-1 yields COS cells that bind CHO–P-selectin. Cell surface expression of CD43, L-selectin, and/or SLe^x was confirmed by fluorescence-activated cell sorting analysis with the appropriate MAbs (data not shown). Moreover, a parallel set of experiments demonstrated that all transfections involving the 3/4FT gene yielded COS cells that bound to CHO–E-selectin (data not shown). These results indicate that the protein backbone is an im-

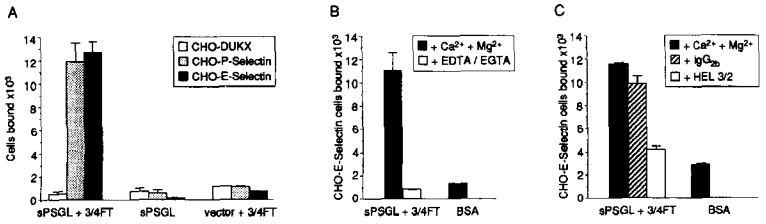

Figure 5. Adhesion of CHO–P-Selectin and CHO–E-Selectin Cells to a Soluble Form of PSGL-1

(A) Conditioned media obtained from COS transfectants (as indicated on the x axis) were diluted 2-fold, coated on plates, and assayed for CHO–P-selectin, CHO–E-selectin, and CHO–DUKX binding. (B and C) CHO–E-selectin binding measured in the presence of EDTA/EGTA (B) and a neutralizing MAb, HEL3/2 (C).

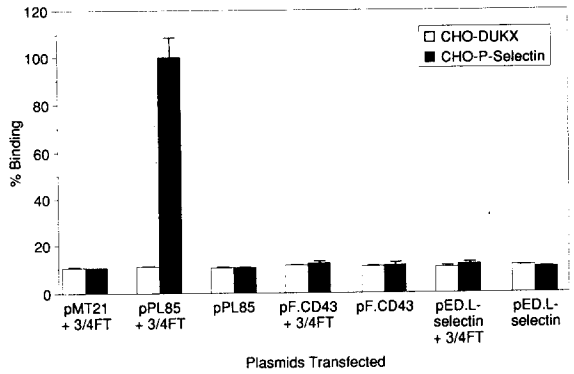

Figure 6. Adhesion of CHO–P-Selectin to COS Cells Expressing Neutrophil Sialoglycoproteins

6-Carboxyfluorescein diacetate–labeled CHO–DUKX (open bars) or CHO–P-selectin (closed bars) cells were added to wells containing adherent COS cells transfected as indicated.

portant component in defining PSGL-1 as a P-selectin ligand.

Effect of Glycosidase Digestions on sPSGL-1.T7

sPSGL-1.T7, derived from cotransfected COS cells and affinity purified with immobilized $LEC_{\gamma 1}$, was digested with various glycosidases and evaluated for alterations in electrophoretic mobility after immunoprecipitation with a polyclonal antibody (Figure 7, lanes labeled "A"). The polyclonal antibody was generated by immunizing rabbits with an E. coli fusion protein containing the N-terminal one-third of PSGL-1 linked to maltose-binding protein. As shown in Figure 7 (lane 2), this antibody was able to precipitate

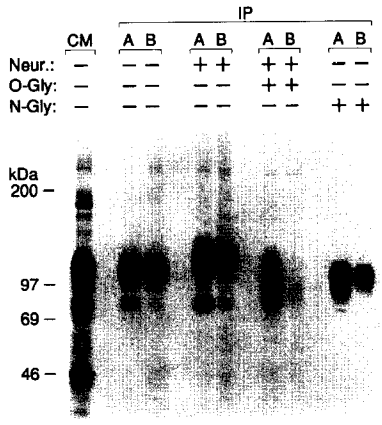

Figure 7. Glycosidase Digestions of Soluble P-Selectin Glycoprotein Ligand

COS-conditioned medium containing [^{35}S]methionine-labeled sPSGL-1.T7 was precipitated with $LEC_{\gamma 1}$ and digested with glycosidases as described by Dorner and Kaufman (1990). For each glycosidase treatment, lanes labeled "A" represent digest samples reprecipitated with a polyclonal antibody to an E. coli fusion protein containing a portion of PSGL-1, and lanes labeled "B" correspond to digest samples reprecipitated with $LEC_{\gamma 1}$. Lane 1, nonprecipitated COS-conditioned medium; lanes 2 and 3, untreated sPSGL-1.T7; lanes 4 and 5, neuraminidase treated; lanes 6 and 7, neuraminidase and O-glycanase treated; lanes 8 and 9, N-glycanase treated.

radiolabeled and affinity-purified sPSGL-1.T7. In parallel, the effect of glycosidase treatment upon P-selectin binding was evaluated after digestion by reprecipitation with $LEC_{\gamma 1}$ (Figure 7, lanes labeled "B").

Treatment of affinity-purified sPSGL-1.T7 with neuraminidase resulted in a decreased mobility on SDS–PAGE (Figure 7, lane 4) and a partial reduction in the amount of ligand recaptured with $LEC_{\gamma 1}$ (Figure 7, lane 5). The electrophoretic mobility change is consistent with a reduction in the net charge of the molecule due to removal of the negatively charged sialic acid residues (Carlsson and Fukuda, 1986). Digestion with both neuraminidase and O-glycanase caused an increase in electrophoretic mobility (Figure 7, lane 6) and essentially complete abrogation of P-selectin binding (Figure 7, lane 7). Digestion with N-glycanase also resulted in an increased electrophoretic mobility (Figure 7, lane 8) and a significant reduction in the amount of ligand recaptured with $LEC_{\gamma 1}$ (Figure 7, lane 9). These data indicate that the PSGL-1.T7 is extensively glycosylated with both N- and O-linked oligosaccharides and that a portion of the apparent discrepancy in molecular mass can be attributed to the presence of carbohydrate. Moreover, these data suggest that sialylated oligosaccharides and particularly O-linked oligosaccharides are crucial components of the P-selectin binding determinant(s) on sPSGL-1.T7.

A Polyclonal Antibody against COS sPSGL-1.T7 Recognizes an HL-60 Membrane Protein That Specifically Binds P-Selectin

Radiolabeled glycoprotein ligands for P-selectin were purified from both HL-60 cells and cotransfected COS cells as described in Experimental Procedures. Three equal aliquots of each cell preparation were then subjected to immunoprecipitation, SDS–PAGE, and autoradiography as follows. Reprecipitation of each preparation with $LEC_{\gamma 1}$ (Figure 8, lanes 5 and 6) yielded a single major species in both extracts that exhibited an approximate molecular mass of 220 kd (nonreducing conditions) or 110 kd (reducing conditions; data not shown). Immunoprecipitation with a neutralizing rabbit polyclonal antibody (Rb3026), generated by immunization with sPSGL-1.T7 from cotransfected COS cells, results in a banding pattern for each extract identical to that observed upon $LEC_{\gamma 1}$ precipitation (Figure 8, lane 1 versus lane 5, lane 2 versus lane 6). No bands were observed for either extract when preimmune rabbit serum is substituted for the polyclonal antibody (Figure 8, lanes 3 and 4). These results demonstrate that the major P-selectin glycoprotein ligand from HL-60 cells is also homodimeric and immunocrossreactive with sPSGL-1.T7.

Discussion

This report describes the isolation of a cDNA that encodes the protein component of a P-selectin glycoprotein ligand (PSGL-1), a mucin-like transmembrane glycoprotein present on myeloid cells that functions as a ligand for P-selectin when appropriately glycosylated. PSGL-1 was identified by a novel expression cloning method that utilized the

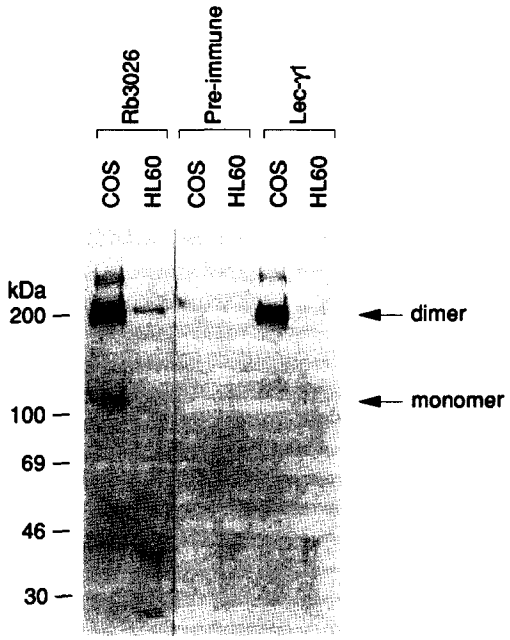

Figure 8. A Polyclonal Antibody against COS-Produced PSGL-1 Recognizes a Specific Protein from HL-60 Cells

P-selectin binding proteins were affinity purified with $LEC_{\gamma 1}$ from membrane extracts of [^{35}S]methionine-labeled HL-60 cells or cotransfected COS cells. Bound material from cell extracts was then reprecipitated with either polyclonal antibody (Rb3026) raised against sPSGL-1.T7, preimmune sera, or $LEC_{\gamma 1}$.

cotransfection of an α(1,3/1,4)fucosyltransferase (3/4FT) gene during the screening of an HL-60 cDNA library. PSGL-1 was characterized as a homodimeric glycoprotein of 220 kd that exhibits the same binding characteristics toward P-selectin as neutrophils or HL-60 cells; binding was dependent upon the presence of divalent cations and could be blocked by a neutralizing anti-P-selectin antibody. Cotransfected COS cells and a soluble form of PSGL-1 (sPSGL-1.T7) also possess these P-selectin binding characteristics, the latter suggesting that these binding properties can be ascribed solely to PSGL-1. Cotransfection with plasmids encoding other neutrophil sialoglycoproteins yields COS cells incapable of binding to P-selectin. Southern blot analysis of human genomic DNA indicated a single gene encoding PSGL-1, and RNA blot analysis detected a predominant 2.5 kb mRNA in a number of human cell lines known to bind P-selectin. Finally, O-linked glycosylation in particular appears to be important for the binding of PSGL-1 to P-selectin. Thus, we conclude that PSGL-1 is the protein component of the major P-selectin glycoprotein ligand of leukocytes.

PSGL-1 appears to be a physiologically relevant ligand for P-selectin, since PSGL-1 mRNA is observed in human cells known to bind P-selectin. Further, a polyclonal antibody raised against sPSGL-1.T7 crossreacts with the major P-selectin binding protein isolated from HL-60 cells. This protein exhibits comparable molecular weights to PSGL-1 from cotransfected COS cells under both reducing and nonreducing SDS–PAGE. At a minimum, these data suggest that this HL-60 protein shares a common immuno epitope with PSGL-1. It is notable that McEver and colleagues (Moore et al., 1992; Norgard et al., 1993) have previously reported a 240 kd (120 kd reduced) sialoglycoprotein in cell extracts of HL-60 cells and neutrophils by blotting with ^{125}I-P-selectin and by affinity chromatography on immobilized P-selectin (Moore et al., 1992). Additional experiments, such as amino acid sequence analysis of the HL-60 ligand, are necessary to establish unequivocally the relationship between this protein and PSGL-1.

As a ligand for P-selectin, PSGL-1 is likely to play a significant role in the initiation of inflammatory and thrombogenic responses in vivo. P-selectin mediates the adhesion of activated platelets or endothelia to neutrophils or monocytes, rapidly translocating from the intracellular granula of platelets or endothelia to the cell surface upon exposure to agents such as thrombin, histamine, or tumor necrosis factor α (Larsen et al., 1989; Weller et al., 1992) and mediating the "rolling" of leukocytes to activated endothelia (Lawrence and Springer, 1991). P-selectin has been demonstrated to play a role in a number of inflammatory and thrombotic disorders, including ischemia–reperfusion injury (Weyrich et al., 1993), leukocyte adherence to lung endothelia in rats infused with cobra venom factor (Mulligan et al., 1992), monocyte adhesion to synovial microvasculature in rheumatoid arthritis (Grober et al., 1993), and leukocyte accumulation in thrombogenic grafts (Palabrica et al., 1992). An understanding of the synthesis, regulation, and structure of PSGL-1 should yield new insights into the mechanisms controlling P-selectin-mediated adhesion.

The observation that PSGL-1 can also serve as a ligand for E-selectin is similar to previous results (Picker et al., 1991) describing neutrophil L-selectin as a ligand for E-selectin. Like L-selectin, PSGL-1 most likely bears the SLe^x moiety that can mediate E-selectin binding. Thus, simple presentation of SLe^x may be sufficient to mediate E-selectin binding, regardless of the "scaffolding" (i.e., lipid or protein) employed for its presentation. Our results showing significant binding of CHO–E-selectin cells to all COS cell transfectants expressing 3/4FT are consistent with this possibility. In contrast, the recent report of a specific glycoprotein ligand for murine E-selectin (Levinovitz et al., 1993) suggests that the physiological ligand for human E-selectin may be a subset of all the glycoproteins containing SLe^x. If so, it is not clear what role, if any, the polypeptide of such ligands plays in derining binding specificity to E-selectin. However, our results clearly demonstrate that the polypeptide is critical in defining PSGL-1 as a ligand for P-selectin. Thus, it is interesting to note that Picker et al. (1991) found that an anti-L-selectin MAb blocked the binding of PMNs to COS cells expressing P-selectin. Since our results suggest that L-selectin is itself not a ligand for P-selectin, other roles for L-selectin in P-selectin-mediated adhesion events are still possible but will require further experimentation.

Experimental Procedures

P-Selectin Chimera ($LEC_{\gamma 1}$) and α(1,3/1,4)Fucosyltransferase

A chimeric soluble form of P-selectin, termed $LEC_{\gamma 1}$, was made by

fusing the extracellular portion of P-selectin and the Fc portion of human IgG1 using conventional recombinant DNA techniques.

The α(1,3/1,4)fucosyltransferase (3/4FT) gene (Kukowska-Latallo et al., 1990) was cloned from total human genomic DNA (Clontech Laboratories) by means of polymerase chain reaction. The gene was identified by DNA sequencing and placed into the COS expression vector pEA.

P-Selectin Glycoprotein Ligand Expression Cloning and Sequence Analysis

Human HL-60 cDNA was ligated into the EcoRI cloning site of vector pMT21, a modified form of the expression vector pMT2 (Bonthron et al., 1986). In the first stage of cloning, a "panning" technique (Aruffo and Seed, 1987) was utilized to enrich for the plasmid of interest. Plasmids from the cDNA library were coexpressed with pEA.3/4FT in COS cells. At approximately 45 hr posttransfection, the COS cells were suspended by treatment with 1 mM EGTA in phosphate-buffered saline and panned over plates coated with $LEC_{\gamma 1}$ captured by an anti-human IgG1 Fc polyclonal antibody (Jackson ImmunoResearch). Plasmids from adherent cells were rescued (Hirts, 1967) and amplified for subsequent rounds of expression and panning until a pool exhibited significantly higher binding, relative to background, to the $LEC_{\gamma 1}$-coated plate. This pool was subdivided and screened by a cell–cell adhesion assay employing the binding of 6-carboxyfluorescein diacetate-labeled (Brenan and Parish, 1984) CHO–P-selectin cells to the transfected COS cells. Positive subpools were identified by fluorescence microscopy and further subdivided and screened until an individual plasmid (pPL85) was identified as a positive clone.

Protein data base searches were performed using the FASTA program with the PIR, SwissProt, and GenPept data bases. DNA data base searches were performed using the BLASTN program with the GenBank and EMBL data bases. Data base searches for the repeat sequences of PSGL-1 were performed using the FindPatterns program (Devereux et al., 1984).

Adhesion Assays

Transfected COS cells were trypsinized and transferred into 12- or 24-well tissue culture plates at 24 hr posttransfection. Suspended CHO–P-selectin cells labeled with 6-carboxyfluorescein diacetate or [^{3}H]thymidine (New England Nuclear) were incubated in either binding buffer (Tris-buffered saline + 1% bovine serum albumin, 1% fetal calf serum, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, 0.01% aprotinin) or chelating buffer (binding buffer + 3 mM each of EDTA and EGTA) prior to adding to the adherent COS cells. The adhesion assay was performed as described previously (Larsen et al., 1992). Bound cells were quantitated by a microplate fluorometer (Cambridge Technology, Inc.) or a scintillation counter. In adhesion assays employing COS-conditioned medium, the serum-free medium was coated onto 48-well plates. [^{3}H]thymidine-labeled CHO cells were added to the bovine serum albumin–blocked wells, and binding was quantitated.

Protein Blot Analysis

Transfected COS cells were suspended in "relaxation buffer" (Moore et al., 1992) and lysed by sonication. A membrane fraction was prepared from postnuclear supernatant by high speed centrifugation (100,000 × g). The pellet was extracted and analyzed by blotting as described previously (Moore et al., 1992) with the following modifications: $LEC_{\gamma 1}$ or a human IgG1 (Sigma) was preincubated with ^{125}I-protein A (Amersham) (+/-3 mM EGTA and 3 mM EDTA or MAbs) for 30 min. The mixture was then incubated with the blots for 60 min, and the washed filters were subjected to autoradiography.

Precipitation of Proteins from COS and HL-60 Membrane Extracts

HL-60 cells or transfected COS cells were metabolically labeled with [^{35}S]methionine, and cell membrane extracts were prepared as described above. The detergent extract was diluted 5-fold with IP buffer (20 mM Tris [pH 7.2], 150 mM NaCl, 2 mM $CaCl_2$, 5 mg/ml bovine serum albumin, 0.02% NaN_3) and incubated for 1 hr at 4°C with $LEC_{\gamma 1}$ preabsorbed onto protein A–Sepharose. After extensive washing, bound material was eluted with a buffer containing EDTA (20 mM Tris [pH 7.2], 5 mM EDTA, 150 mM NaCl).

Southern and RNA Blot Analysis

Southern blot analysis was performed using standard procedures (Sambrook et al., 1989) with a human genomic DNA blot (Clontech, Palo Alto, CA). For Northern analysis, HL-60, THP-1, U937, and HepG2 poly(A)$^+$ RNA were isolated using standard procedures (Sambrook et al., 1989). Total RNA was extracted from human monocytes isolated from peripheral blood mononuclear cells by adherence to plastic and from human PMNs isolated from whole blood by centrifugation over a Ficoll–Hypaque density gradient (Mono-Poly Resolving Medium, ICN Biomedical). Agarose–formaldehyde gel electrophoresis in MOPS buffer of RNA samples was carried out essentially as described (Sambrook et al., 1989). Filters were hybridized with a ^{32}P-labeled cDNA probe for PSGL-1 comprising nucleotides 60–389 and washed in 2 × SSC (low stringency) or 0.3 × SSC (high stringency), in the presence of 0.1% SDS at 65°C.

Construction and Expression of Soluble PSGL-1

The plasmid DNA encoding the soluble, extracellular form of the P-selectin ligand was constructed as follows: pPL85 plasmid DNA was restricted with XbaI and HincII, and the approximately 950 bp fragment containing all of the extracellular segment of the PSGL-1 gene up to and including the codon for valine 295 was gel isolated. Oligonucleotides encoding 14 amino acids, including an epitope derived from the phage T7 major capsid protein, were synthesized, creating a C-terminal fusion of the epitope "tag" with an additional 32 amino acids derived from the vector sequence of expression plasmid pED (Kaufman et al., 1991). The resulting plasmid is pED.sPSGL-1.T7.

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